with a concentration in the range of 1.240.15 IU/ml using PBS/BSA solution as diluent. If necessary, adjust the starting dilution to obtain responses falling in the linear portion of the dose-response curve.

Reference solutions. Reconstitute the reference preparation according to instructions. Prepare at least 3 independent replicates of at least 3 serial 1.5 or two-fold dilutions starting with a concentration in the range of 1.240.15 IU/ml using PBS/BSA solution as diluent. If necessary, adjust the starting dilution to obtain responses falling in the linear portion of the dose-response curve.

Distribute 50 µl of the D-positive red blood cells into each well of a microtitre plate. Add 50 µl of each of the dilutions of the test solution or reference solution to each of a series of wells. Use 50 µl of PBS-BSA solution as negative control. Distribute 50 µl of the D-negative red blood cells into 4 wells of the same microtitre plate and add 50 µl of the lowest dilution of the test preparation. To monitor spurious reactions distribute 50 µl of the D-positive red blood cells into 4 wells of the same microtitre plate and add 50 µl of PBS-BSA solution. Seal with plastic film and incubate at 37 °C for 40 min.

Centrifuge the plates at 50 g for 3 min, discard the supernatant and wash the cells with 200-250 µl of PBS-BSA solution. Repeat this at least once. Centrifuge the plates at 50 g for 3 min, discard the supernatant and add 50 µl of the secondary antibody diluted with PBS-BSA solution to a suitable protein concentration. Seal with plastic film and incubate, protected from light, at room temperature for 20 min.

Centrifuge the plates at 50 g for 3 min, discard the supernatant and wash the cells with 200-250 µl of PBS-BSA solution. Repeat this at least once. Centrifuge the plates at 50 g for 3 min, resuspend the cells into 200-250 µl of PBS. Transfer the cell suspension into a tube suitable for the flow cytometry equipment available and further dilute by adding PBS to allow a suitable flow rate. Proceed immediately with measurement of the median fluorescence intensity in a flow cytometer. Record at least 10 000 events without gating but excluding debris.

Use the median fluorescence intensity in the linear range of the dose response curve to estimate the potency of the preparation to be examined by the usual statistical methods, (5.3).

**Determination of potency of the vaccine to be examined.** Using a 9 g/1 solution of sodium chloride R containing the aluminium adjuvant used for the vaccine, prepare at least three dilutions of the vaccine to be examined and matching dilutions of the reference preparation. Allocate the dilutions one to each of the groups of animals and inject subcutaneously not more than 1.0 ml of each dilution into each animal in the group to which that dilution is allocated. Maintain a group of unvaccinated controls, injected subcutaneously with the same volume of diluent. After 28 to 32 days, anaesthetise and bleed all animals, keeping the individual sera separate. Assay the individual sera for specific antibodies against hepatitis B virus by a suitable immunochemical method (2.7.1).

**Calculations.** Carry out the calculations by the usual statistical methods for an assay with a quantal response (5.3).

From the distribution of reaction levels measured on all the sera in the unvaccinated group, determine the maximum reaction level that can be expected to occur in an unvaccinated animal for that particular assay. Any response in vaccinated animals that exceeds this level is by definition a seroconversion.

Make a suitable transformation of the percentage of animals showing seroconversion in each group (for example, a probit transformation) and analyse the data according to a parallel-line log dose-response model. Determine the potency of the test preparation relative to the reference preparation.

**Validity conditions.** The test is not valid unless:
- for both the test and the reference vaccine, the ED₅₀ lies between the smallest and the largest doses given to the animals,
- the statistical analysis shows no significant deviation from linearity or parallelism,
- the confidence limits (P = 0.95) are not less than 33 per cent and not more than 300 per cent of the estimated potency.

**Potency requirement.** The upper confidence limit (P = 0.95) of the estimated relative potency is not less than 1.0.

**IN VITRO ASSAY**

Carry out an immunochemical determination (2.7.1) of antigen content with acceptance criteria validated against the in vivo test. The acceptance criteria are approved for a given reference preparation by the competent authority in the light of the validation data.

**Hepatitis A vaccine (inactivated, adsorbed) type A BRP, hepatitis A vaccine (inactivated, adsorbed) type B BRP and hepatitis A vaccine (inactivated, adsorbed) type C BRP** are suitable for the in vitro assay of certain vaccines as described in the accompanying leaflet.

**2.7.14. ASSAY OF HEPATITIS A VACCINE**

The assay of hepatitis A vaccine is carried out either in vitro, by comparing in given conditions its capacity to induce specific antibodies in mice with the same capacity of a reference preparation, or in vivo, by an immunochemical determination of antigen content.

**IN VIVO ASSAY**

The test in mice shown below is given as an example of a method that has been found suitable for a given vaccine; other validated methods may also be used.

**Selection and distribution of the test animals.** Use in the test healthy mice from the same stock, about 5 weeks old and from a strain shown to be suitable. Use animals of the same sex. Distribute the animals in at least 7 equal groups of a number suitable for the requirements of the assay.

**2.7.15. ASSAY OF HEPATITIS B VACCINE (rDNA)**

The assay of hepatitis B vaccine (rDNA) is carried out either in vitro, by comparing in given conditions its capacity to induce specific antibodies against hepatitis B surface antigen (HBsAg) in mice or guinea-pigs with the same capacity of a reference preparation, or in vivo, by an immunochemical determination of the antigen content.
IN VIVO ASSAY

Selection and distribution of the test animals. Use in the test healthy mice from the same stock, about 5 weeks old. The strain of mice used for this test must give a significant slope for the dose-response curve to the antigen; mice with haplotype H-2\textsuperscript{d} or H-2\textsuperscript{v} are suitable. Healthy guinea-pigs weighing 300 g to 350 g (about 7 weeks old) from the same stock are also suitable. Use animals of the same sex. Distribute the animals in at least 7 equal groups of a number appropriate to the requirements of the assay.

Determination of potency of the vaccine to be examined. Using a 9 g/1 solution of sodium chloride \( R \) containing the aluminium adjuvant used for the vaccine or another appropriate diluent, prepare at least three dilutions of the vaccine to be examined and matching dilutions of the reference preparation. Allocate the dilutions one to each of the groups of animals and inject intraperitoneally not more than 1.0 ml of each dilution into each animal in the group to which that dilution is allocated. One group of animals remains unvaccinated and is injected intraperitoneally with the same volume of diluent. After an appropriate time interval (for example, 4 to 6 weeks), anaesthetise and bleed the animals, keeping the individual sera separate. Assay the individual sera for specific antibodies against HBsAg by a suitable immunochemical method (2.7.1).

Calculations. Calculations are carried out by the usual statistical methods for an assay with a quantal response (5.3).

From the distribution of reaction levels measured on all the sera in the unvaccinated group, the maximum reaction level that can be expected to occur in an unvaccinated animal for that particular assay is determined. Any response in vaccinated animals that exceeds this level is by definition a seroconversion.

Make a suitable transformation of the percentage of animals showing seroconversion in each group (for example, a probit transformation) and analyse the data according to a parallel-line log dose-response model. Determine the potency of the test preparation relative to the reference preparation.

Validity conditions. The test is not valid unless:

- for both the test and the reference vaccine, the \( \text{ED}_{50} \) lies between the smallest and the largest doses given to the animals,
- the statistical analysis shows no significant deviation from linearity or parallelism,
- the confidence limits (\( P = 0.95 \)) are not less than 33 per cent and not more than 300 per cent of the estimated potency.

Potency requirement. The upper confidence limit (\( P = 0.95 \)) of the estimated relative potency is not less than 1.0.

IN VITRO ASSAY

Carry out an immunochemical determination (2.7.1) of antigen content with acceptance criteria validated against the in vitro test.

Enzyme-linked immunosorbent assay (ELISA) and radio-immunooassay (RIA) using monoclonal antibodies specific for protection-inducing epitopes of HBsAg have been shown to be suitable. Suitable numbers of dilutions of the vaccine to be examined and the reference preparation are used and a parallel-line model is used to analyse the data which may be suitably transformed. Kits for measuring HBsAg in vitro are commercially available and it is possible to adapt their test procedures for use as an in vitro potency assay.

The acceptance criteria are approved for a given reference preparation by the competent authority in the light of the validation data.

Hepatitis B vaccine (rDNA) method A BRP and hepatitis B vaccine (rDNA) method B BRP are suitable for the in vitro assay of certain vaccines as described in the accompanying leaflet.

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2.7.16. ASSAY OF PERTUSSIS VACCINE (ACELLULAR)

The capacity of the vaccine to induce the formation of specific antibodies is compared with the same capacity of a reference preparation examined in parallel; antibodies are determined using suitable immunochemical methods (2.7.1) such as enzyme-linked immunosorbent assay (ELISA). The test in mice shown below uses a three-point model but, after validation, for routine testing a single-dilution method may be used.

Reference vaccine. A batch of vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The stability of the reference vaccine shall be documented.

Reference antiserum. Bordetella pertussis mouse antiserum BRP is suitable for use as a reference antiserum.

Requirement. The capacity of the vaccine to induce antibodies is not significantly (\( P = 0.95 \)) less than that of the reference vaccine.

The following test model is given as an example of a method that has been found to be satisfactory.

Selection and distribution of test animals. Use in the test healthy mice (for example, CD1 strain) of the same stock, about 5 weeks old. Distribute the animals in 6 groups of a number appropriate to the requirements of the assay. Use 3 dilutions of the vaccine to be examined and 3 dilutions of a reference preparation and attribute each dilution to a group of mice. Inject intraperitoneally or subcutaneously into each mouse 0.5 ml of the dilution attributed to its group.

Collection of serum samples. 4 to 5 weeks after vaccination, bleed the mice individually under anaesthesia. Store the sera at \(-20 °\text{C}\) until tested for antibody content.

Antibody determination. Assay the individual sera for content of specific antibodies to each component using a validated method such as the ELISA test shown below.

ELISA test. Microtitre plates (polyvinyl chloride) or polystyrene as appropriate for the specific antigen) are coated with the purified antigen at a concentration of 100 ng per well. After washing, unreacted sites are blocked by incubating with a solution of bovine serum albumin and then washed. Two-fold dilutions of sera from mice immunised with test or reference vaccines are made on the plates. After incubation at 22-25 °C for 1 h, the plates are washed. A suitable solution of anti-mouse IgG enzyme conjugate is added to each well and incubated at 22-25 °C for 1 h. After washing, a chromogenic substrate is added from which the bound enzyme conjugate liberates a chromophore which can be quantified by measurement of absorbance (2.2.25). The test conditions are designed to obtain a linear response for absorbance with respect to antibody content over the range of measurement used and absorbance values within the range 0.1 to 2.0.